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# Nanoformulations of a Triazene Analogue with Specific Affinity to Human Melanoma

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## Abstract

Malignant melanoma remains an aggressive malignancy conferring a very poor prognosis and standard treatments have not demonstrated an overall survival benefits. The alkylating agents triazenes have been used for treatment of malignant melanoma. However, their lack of specificity toward tumor cells requires the urgent development of novel therapeutic strategies. Therefore, the main objectives of the present work were the use of a triazene prodrug, previously synthesized and adequately formulated in liposomes. This triazene prodrug has demonstrated in previous work to be a good substrate for tyrosinase, an enzyme that is overexpressed in melanoma cells. After optimization of liposome preparation conditions and selection of most appropriated lipid compositions, it was possible to achieve high incorporation efficiencies. The association of this prodrug in liposomes, primarily relies in the possibility of solving solubility problems, increasing internalization in melanoma cell lines, protecting the molecule from premature degradation and enhancing its therapeutic index. Prodrug in free and liposomal forms demonstrated a high stability in the presence of human plasma, at 37°C. The *in vitro* tests, performed in human melanoma cell lines, demonstrated that the incorporation of TPD in liposomes was able to potentiate the cytotoxic effect of this triazene prodrug. The $IC_{50}$ for the prodrug in free form was superior to 125 μM, whereas in the liposomal form this value ranged from 13 to 22 μM being dependent on the lipid composition. The obtained results represent an excellent approach to fight malignant melanoma.

**Keywords:** Malignant melanoma, Liposomes, Triazene analogues, Tyrosinase, Melanoma cells, *In vitro* tests.

## Introduction

Malignant melanoma is the most aggressive form of skin cancer. The incidence of this type of cancer varies from 3-5/100 000/year in Mediterranean region to 12-20 in Nordic countries and it has been increasing over the years, being more common in women than in men [1]. Although representing only 11% of skin cancers, malignant melanoma persists as the major cause of death, more than 90%, due to inadequate therapies [2].

When detected in early stages may be curable, if surgically removed, but in later stages is very difficult to treat due to high metastization. Although early diagnosis remains the best method to fight melanoma, the options for medical treatment in more advanced tumor stages include chemotherapy, radiation, and immunotherapy [2] Chemotherapy typically includes the administration of dacarbazine (DTIC), cisplatin, temozolomide (TMZ), tamoxifen or paclitaxel [3]. Systemic immunotherapy includes interferon-α and interleukin-2 [4]. Nevertheless, none of these treatments have improved long term efficacy in more than 15-35% of patients, and all with moderate to significant toxicities and side effects [5]. In 2011 U.S. Food and Drug Administration (FDA) and European Medicines Agency (EMA), have approved two new drugs, ipilimumab and vemurafenib. Ipilimumab is a monoclonal antibody that activates the immune system by targeting CTLA-4, a protein receptor that downregulates the immune system [6]. Vemurafenib was the first oral molecularly targeted therapy to be licensed in the US and Europe specifically indicated for patients with melanoma whose tumors have V600E mutations in the BRAF gene by blocking the function of the V600E mutated BRAF protein. Vemurafenib and Ipilimumab have contributed to a small increase in therapeutic responses, but the extensive costs, the severe side effects, the emergence of resistances and development of skin lesions such as keratoacanthomas [7] led us to consider the urgent need of novel therapies and/or novel drug delivery systems.

Triazenes are a well-known class of DNA alkylating agents for the treatment of malignant melanoma. This name is derived from its chemical structure consisting of three consecutive nitrogen atoms, which are in most of cases adjacent to an aromatic ring. Examples of triazene derivatives with clinical application in the treatment of malignant melanoma are DTIC and TMZ as above mentioned. The main problems of these molecules are their lack of specificity, therapeutic resistance and the frequency of side-effects leading to disappointing therapeutic benefits [2].

In order to overcome the clinical limitations of DTIC and TMZ,

several strategies have been the subject of study by chemists and biologist throughout the years. One of these strategies has been the development of selective prodrugs that can specifically target malignant melanoma cells [8-10]. Based on the knowledge of the up-regulation of tyrosinase in melanoma cells, several prodrugs with close structural resemblances with natural tyrosinase substrates where synthesized, their stability evaluated as well as the cytotoxicity in melanoma cells with different levels of tyrosinase activity [11-13]. However, besides the development of appropriate prodrugs presenting high specificity to tyrosinase in *in vitro* tests, when transposing to *in vivo*, in order to achieve a pharmacological effect, they have to reach tumor sites at therapeutic concentrations. The therapeutic performance of these molecules will be dependent on several factors: biodistribution profile, drug solubility, degradation before reaching tumor sites, route of administration, toxicity derived from localization in non-affected sites. As a way of overcoming some of above mentioned disadvantages the incorporation of tyrosinase substrates in appropriate delivery system, liposomes, constitutes the main objective of the present work. Liposomes as drug delivery system are able to alter the biodistribution profile of the associated drug improving the overall pharmacological properties of commonly used chemotherapeutics [14-16]. The ease of how they are generated and modified turns them very useful for the treatment of a wide variety of cancers.

The main objective of the present work was the construction of liposomes for incorporating a triazene prodrug (TPD) previously synthesized and characterized [13] to be further tested in a murine melanoma model. The influence of different lipid compositions on the TPD incorporation parameters, stability in biological fluids and cytotoxic effect against a human melanoma cell line was investigated.

## Materials

Pure phospholipids, egg phosphatidylcholine (PC), phosphatidylglicerol (PG), dimyristoyl phosphatidylcholine (DMPC), dimyristoyl phosphatidylglicerol (DMPG) and distearoyl phosphatidylethanolamine covalently linked to poly (ethylene glycol) 2000 (PEG), used for the preparation of liposomal formulations were purchased from Avanti Polar Lipids (Alabaster, AL). Deionized water (Milli-Q system; Millipore, Tokio) was used for the preparation of solutions in all experiments. Nuclepore Track-Etch Membranes were purchased from Whatman Ltd, NY, USA. Culture media and antibiotics from Gibco were obtained from Invitrogen, Life Technologies Corporation, (NY, USA). Reagents for cell proliferation assays were obtained from Promega, (Madison, WI, USA). Melanoma cell line MNT-1 was kindly provided by Doctor Paula Videira from the Faculty of Medical Sciences of the New University of Lisbon.

## Methods

The triazene prodrug (TPD) was prepared by linking the amino acyl derivative of triazene to the tyrosinase substrate, the N-acyl-tyrosine as previously described [13].

### TPD liposomal formulations

Multilamellar liposomes composed of the selected phospholipids were prepared by the dehydration-rehydration method (DRV) [17-19]. Briefly, the selected phospholipids, at 30 μmol/mL together with the TPD, were dissolved in chloroform and solvent evaporated by rotary evaporation(Buchi, Switzerland) to obtain a thin film under a nitrogen stream. This film was dispersed in deionized water, frozen in liquid nitrogen and lyophilized (Freeze dryer, Edwards, USA) overnight.

The HEPES buffer, pH 7.4 (10 mM, 145 mM NaCl), was then added to the lyophilized powder, up to two-tenth of the volume of the original dispersion. This hydration step lasts 30 min and, subsequently, HEPES buffer, pH 7.4, was added up to the starting volume. The temperature of the hydrating medium was always above the gel-liquid crystal transition temperature (Tc) of the phospholipid.

In order to homogenize and reduce the mean size of liposomes, the so formed suspensions were submitted to an extrusion step through polycarbonate filters of appopriate pore size until the desire vesicle size is rechead (0.8, 0.6, 0.4 (2x) and 0.2 (3x) μm) under a nitrogen pressure of 100-500 $lb/in^2$ with an Extruder device (Lipex: Biomembranes Inc., Vancouver, British Columbia, Canada).

The separation of non incorporated TPD was perfomed by a gel filtration (Econo-Pac® 10DG; Bio-Rad Laboratories, Hercules, CA) followed by ultracentrifugation at 250,000 g for 120 min at 15°C in a Beckman LM-80 ultracentrifuge (Beckman Instruments, Inc, USA.) Finally, the pellet was ressuspended in HEPES buffer, pH 7.4.

### TPD quantification

The quantification of TPD was performed by HPLC according to [20] with some modifications.

### HPLC System

The HPLC system was an ELITE LaChrom Hitachi (Japan), comprising: an L-2130 pump module, a Diode-Array L-2455 detector and an autosampler L-2200 with a loop of 20 μL. The wavelenght of the detector was set at 300 nm. The system was connected to a computer with specific software, Ez Chrom Elite, for integration and treatment of chromatograms. The analytical column was a LiChroCART® (150-4,6) Purospher® Star RP-18 (5 μm) (Merck) equipped with the respective guard-column. The mobile phase, in an isocratic solvent system, consisted on acetonitrile (ACN)/water ($H_2O$) (50:50 (v/v)) with a flow rate of 0.7 mL/min at 25°C.

### Preparation of standard solutions

Stock solutions (0.5 mM) of TPD and of the respective hydrolysis products monomethyltriazene (MMT) and amine were prepared by weighing the appropriate amount of each product and dissolving it in ACN. Different dilutions were made by diluting the respective initial stock solution with ACN. At least three-five-point independent calibration curves ranging from 2 to 16 μM for TPD and from 5 to 25 μM for MMT and amine were used. An intermediate standard for all products was always injected together with the analyzed samples to verify the precision of the obtained chromatograms from their peak area and concentration response.

Samples were appropriately diluted in the range as the respective calibration curves. All mobile phase solvents, standards and samples were filtered through 0.2 μm porosity membranes before injection onto HPLC.

### Characterization of TPD liposomal formulations

TPD content in liposomes was determined by HPLC after disruption of liposomes with ACN. Lipid content of all liposomal formulations was determined using the method described by Rouser and co-workers [21]. TPD liposomes were characterized in terms of incorporation efficiency (I.E.) according to the following equations:

initial TPD to lipid ratio - (TPD/Lip)i;

final TPD to lipid ratio - (TPD/Lip)f;

incorporation efficiency (I.E.) (%) - [(TPD/Lip)f] / [(TPD/Lip) i] x 100

The I.E., being a ratio between final to initial (TPD/Lip), was the measure of efficiency of an available lipid mixture to incorporate hydrophobic TPD in the final liposomal form.

TPD liposomes were also characterized in terms of mean size and polydispersity index (P.I.) by dynamic light scattering and superficial charge by laser Doppler spectroscopy in a ZetaSizer (Malvern Instruments, UK).

### Stability of TPD in free and liposomal forms in human plasma

Stability studies of TPD in free and liposomal forms were performed in human plasma (80% (v/v) diluted in HEPES buffer pH 7.4 and incubated at 37°C), obtained from different healthy individuals and pooled.

The reactions were monitored by HPLC technique which revealed the disappearance of the substrate and the appearance of reaction products. The hydrolysis of TPD in the presence of human plasma gives rise to MMT, which subsequently decomposes in the respective amine.

The hydrolysis reaction was monitored until the determination of the first, second and third half-lives of the prodrug. The mixture was incubated at 37°C and at pre-selected times aliquots of 100 µL were removed from the plasma medium, diluted with 900 µL of ACN, centrifuged and injected onto HPLC column for quantification of TPD, MMT and amine.

### Half-life quantification of TPD

Apparent first-order kinetics and rate constants were determined by using initial rates of hydrolysis [22]. The apparent first-order hydrolysis rate constants of TPD at 37°C were determined by plotting the logarithm of TPD concentration as a function of time according to the equation:

$$\ln(\text{concentration TPD}) = -k_{obs} \times t$$

The hydrolysis half-live (in minutes) was then calculated by the equation:

$$t_{1/2} = \text{Ln}(2) / k_{obs}$$

### Melanoma cell line

The melanoma cell line used was the MNT-1, which was grown in complete culture medium consisting of DMEM supplemented with 10% fetal bovine serum, 2 mM L-glutamine and 100 µg/mL penicillin/streptomycin at 37°C with humidified atmosphere with 5% $CO_2$.

### Assessment of mitochondrial activity by MTS

Cell viability was evaluated in the absence (control) or presence of increasing concentrations of TPD in free and liposomal forms studied by measuring mitochondrial activity, based on the colorimetric method of reducing the compound MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) included in the commercial kit, from Promega (Madison, USA). This method consists in the conversion of MTS salt and its reduction, within the mitochondria, to formazan crystals that are soluble in tissue culture. This compound can be spectrophotometrically quantified and the respective absorbance values at 490 nm are proportional to mitochondrial activity, and consequently to the number of living cells in [23].

### Analysis of cell proliferation and viability

For the determination of cytotoxic effect of TPD, in free and liposomal forms, MNT-1 cells at a concentration of 7.5 x $10^3$ cells/mL were placed in 96 well plates (200 µL) for 24 h in culture conditions above specified. After this period, culture medium was removed and adherent cells were treated with TPD in free or in liposomal forms, at a concentration ranging from 7.5 to 125 µM. Unloaded liposomes constituted another control group, using the same lipid concentrations as in loaded liposomes. All tests were performed with 6 samples of each studied concentration. The incubation periods were 24, 48 and 72 h keeping the culture conditions. After this period, the culture medium was removed from all wells and replaced with 100 µL of incomplete culture medium. Subsequently, 20 µL of MTS were added to all wells, agitated, followed by an incubation period of 60 min, under the same culture conditions above mentioned. In addition, 6 wells were prepared without cells (blank) containing the same volumes of incomplete culture medium and MTS solution.

The absorbance was determined in a microplate absorbance reader (Biotek, ELx800, USA) at a wavelength of 490 nm. Subtraction of the blank (without cells) at 490 nm was carried out to all other samples to yield corrected absorbances. Absorbance values were normalized taking into account that cell viability in negative control (MNT-1 cells) was 100%.

The cytotoxic effect was evaluated by determining the percentage of viable/death cells for each TPD studied concentration. Based on these values, the $IC_{50}$ (prodrug concentration that kills 50% of MNT-1 cells) was calculated, according to an equation proposed by Hills and co-workers [24]. For $IC_{50}$ determination, two concentrations, $X_1$ and $X_2$, and the respective cell densities, $Y_1$ and $Y_2$, that correspond to higher or lesser than half cell density in negative control ($Y_0$), were established, according to the following equation:

$$\text{Log } IC_{50} = \text{Log } X_1 + \{[(Y_1 - (Y_0)/2)]/(Y_1 - Y_2)\} \times (\text{Log } X_2 - \text{Log } X_1)$$

Where,

$Y_0/2$ is the half cell density of the negative control;

$Y_1$ is the cell density above $Y_0/2$;

$X_1$ is the concentration corresponding to $Y_1$;

$Y_2$ is the cell density below $Y_0/2$;

$X_2$ is the concentration corresponding to $Y_2$;

The $IC_{50}$ was determined by linear interpolation between $X_1$ and $X_2$.

All data presented are expressed as mean ± standard deviation (S.D.). Statistical analysis was performed using the ANOVA One Single Factor. The acceptable probability for a significant

difference between mean values was $p<0.05$.

## Results and Discussion

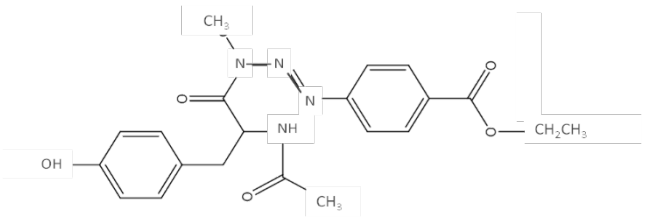
The triazene prodrug studied in the present work was the 3-[(2-(Acetylamino)-3-(4-hydroxyphenylpropanoyl)]-1-(4-ethoxycarbonylphenyl)-3-methyltriazene. The reason for choosing this new synthesized TPD was based on previous results that demonstrated to be good substrate for tyrosinase an enzyme abundant in melanoma cells and being considered an ideal molecular target for the development of anti-melanoma drugs [20,25,26]. Moreover preliminary studies revealed high cytotoxicity against melanoma cell lines [13]. The prodrug is composed by a transporter unit, the N-acetyl-L-tyrosine, which is a good substrate for the tyrosinase, the amide linkage and by the MMT triazene. In Table 1 are shown some physical properties of TPD namely chemical structure, melting point (MP), molecular weight (MW) and octanol/water partition coefficient (Log P). This parameter is a measure of the equilibrium concentration of a solute between two immiscible phases, octanol and water that may predict the potential for partitioning into hydrophobic compartments such as biological membranes and hydrophilic compartments such as blood serum [27]. In a much generalized form we can say that molecules with a Log P higher than 1 present a lipophilic character while solutes with a Log P below 1 are considered hydrophilic. Moreover, Log P > 5 correspond to high lipophilic compounds [28,29].

An optimization of the best experimental conditions using HPLC was performed in order to obtain, in the same chromatogram, the TPD as well as the hydrolysis products: the cytotoxic drug MMT and the respective amine.

In Figure 1 is shown a typical chromatogram resulting from a mixture solution of the three standard compounds using an isocratic solvent system and in Table 2 are shown the retention times for the three compounds.

The optimized HPLC procedure allowed evaluating the TPD stability over time. In Figure 2a is represented the chromatogram of a freshly prepared TPD solution. Along the hydrolysis process we can observe the disappearance of TPD and the increase formation of MMT, Figure 2b, and amine, Figure 2c.

**Table 1:** Physical properties of TPD

| | |
|---|---|
| **Chemical structure** |  |
| **Melting Point (MP)** | 212-215 |
| **Molecular weight (MW)** | 412 |
| **Log P[a]** | 2.96 ± 0.022 |
| **Log P[b]** | 3.16 ± 0.54 |

Data from [20]; **a)** Calculated; **b)** Experimental

Calculated values of Log P were obtained using the ALOGPS 2.1 program

TPD is a low molecular weight molecule with a moderate hydrophobic character presenting a Log P of around 3

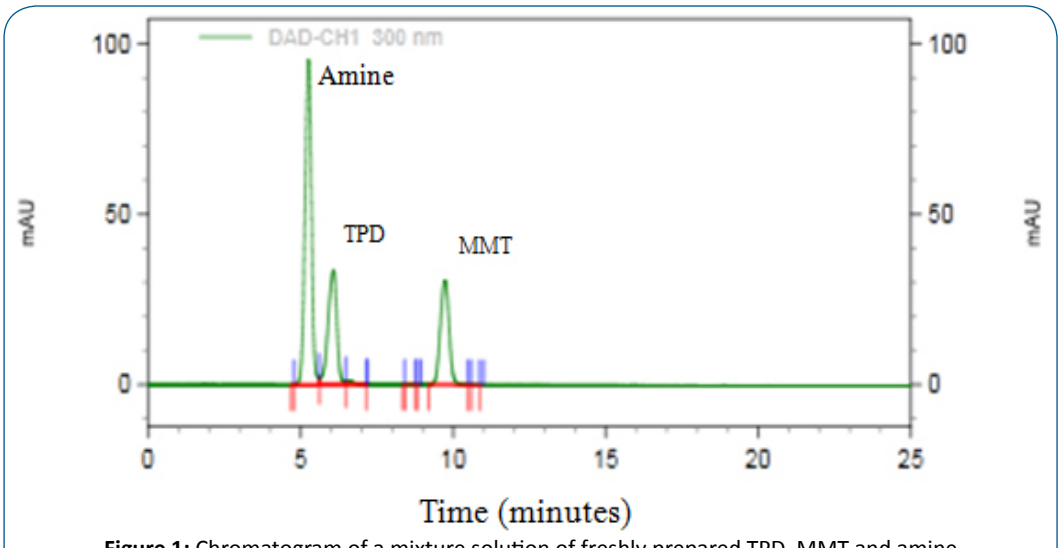



**Figure 1:** Chromatogram of a mixture solution of freshly prepared TPD, MMT and amine

**Table 2:** Retention time for TPD and sub-products

| Compounds | Retention time (min) |
|---|---|
| Amine | 5.1 |
| TPD | 6.1 |
| MMT | 9.8 |

Retention time for the three compounds using an isocratic solvent system. Eluent used - ACN/$H_2O$ (50:50 (v/v)) with a flow rate of 0.7 mL/min

Table 3. They were prepared with neutral phospholipids PC, DMPC or in combination with negatively charged phospholipids PG and DMPG, respectively, which by differing in their phase transition temperatures (Tc), confer to liposomes distinctive membrane fluidities. Formulation 1 was further modified with PEG covalently linked to DSPE (PEG) aiming to achieve a liposomal formulation with long circulating properties.

All TPD liposomal formulations presented mean sizes ranging from 0.16 to 0.18 μm with a polydispersion index (P.I.) below

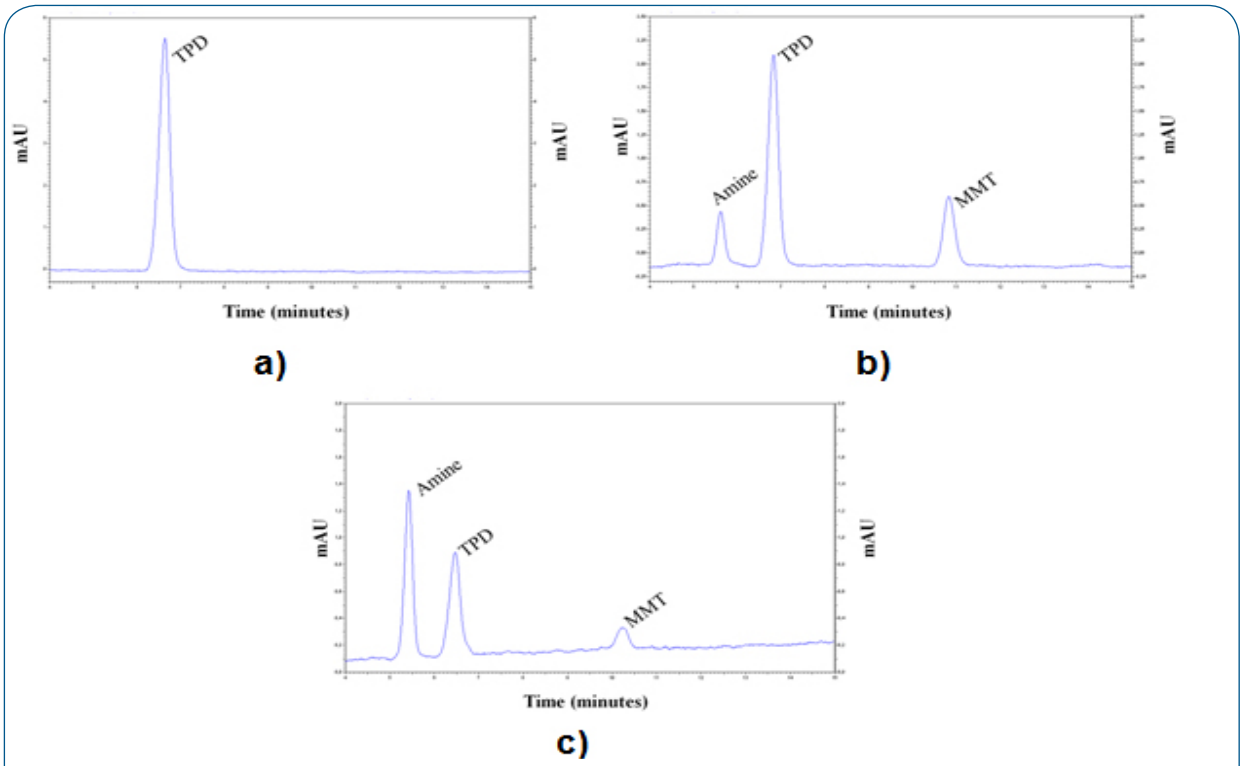



**Figure 2:** Evaluation of TPD hydrolysis over time: **a)** Freshly TPD solution; **b)** and **c)** disappearance of TPD followed by an increase on the formation of MMT and amine

### Incorporation of TPD in liposomes

The selected method for incorporation of TPD in liposomes was the dehydration-rehydration (DRV) method. This method has been used for associating either hydrophilic or hydrophobic molecules resulting in high incorporation parameters [17-19]. Taking into account the Log P obtained for TPD of around 3, as shown in Table 1, this molecule presents a moderate hydrophobic character. Due to this property, it is assumed that TPD may be partially accommodated in the lipid bilayer [28]. For the preparation of liposomes by DRV technique both lipids and TPD were solubilized in an organic solvent, the film hydrated with water and the so formed suspension lyophilized followed by re-hydration in two steps. The first rehydration stage which was performed with two tenth of the original solution volume resulted in a fivefold increase in overall concentration of the solute being this reflected in the concentration of the material that may be entrapped [30]. The rehydration of the lyophilized powder in two steps brings the bilayers and material to be encapsulated into very close contact and the chances for achieving higher incorporation parameters are improved [31].

In the present work 5 liposomal formulations of TPD were developed and the physicochemical properties are shown in

0.2. The low P.I. evidences the high homogeneity of all liposomal formulations. The zeta potential observed for these formulations was in accordance with the charge of the phospholipids used. Formulations 1 and 4 prepared with neutral phospholipids presented a superficial charge close to neutrality, -3 and -4 mV, respectively. For formulation 3 the zeta potential was also in the same range which is accordance with literature [14]. Formulations 2 and 5 that included in the lipid mixture negatively charged phospholipids, PG and DMPG presented zeta potential values of -29 mV, as expected. The incorporation parameters were lipid composition dependent.

The incorporation of TPD in neutral phospholipids (formulation 1 and 4), was dependent on the Tc of the phospholipid used: a reduction on the I.E from 91 to 72% and concomitant final TPD to lipid ratio from 22 to 14 nmol per μmol of lipid was observed. These results are in accordance with published work where the increase on the Tc of the phospholipids used resulted in reduction on incorporation parameters [18,30,33]. The incorporation of an hydrophobic entity in the lipid bilayer that is prepared with fluid phospholipids, such as PC, will allow the accomodation of a higher number of molecules due to the disordered state of the phospholipid acyl chains. On the opposite, the use of more rigid

**Table 3:** Physicochemical properties of TPD liposomes: influence of lipid composition

| Formulation | Lipid composition (molar ratio) [Tc °C] | [TPD/Lip]i (nmol/µmol) | [TPD/Lip]f (nmol/µmol) | I.E. (%) | Ø (µm) (P.I.) | Zeta Pot. (mV) |
|---|---|---|---|---|---|---|
| 1 | PC [-6°C] | 25 ± 2 | 22 ± 1 | 91 ± 8 | 0.21 (<0.2) | -4 ± 1 |
| 2 | PC:PG (7:3) [-6°C:-6°C] | 25 ± 1 | 10 ± 2 | 38 ± 6 | 0.17 (<0.2) | -29 ± 2 |
| 3 | PC:DSPE-PEG (2.85:0.15) [-6°C] | 33 ± 5 | 12 ± 4 | 33 ± 5 | 0.15 (<0.2) | -4 ± 1 |
| 4 | DMPC [+23°C] | 22 ± 3 | 14 ± 3 | 72 ± 17 | 0.19 (<0.2) | -3 ± 1 |
| 5 | DMPC:DMPG (7:3) [+23°C:+23°C] | 26 ± 2 | 3 ± 1 | 13 ± 2 | 0.15 (<0.2) | -29 ± 2 |

Initial lipid concentration [Lip]i – 30 µmol/mL; Initial TPD [TPD]i – 1000 nmol/mL;

I.E. (%) – Incorporation efficiency, [(TPD/Lip)f] / [(TPD/Lip)i] x 100;

Ø – mean size of liposomes; P.I. – polydispersity index; Zeta Pot. – Zeta Potential; [Tc] – phase transition temperature of phospholipids

Values in parentheses correspond to the Tc for each phospholipid from [32]

phospholipids, such as DMPC, presenting higher organized acyl chains will limit the accomodation of hydrophobic molecules in their lipid bilayer [34].

The presence of negatively charged phospholipids in the lipid composition led to lower incorporation parameters being these results more evident for phospholipids with higher Tc. For formulations 2 and 5, the increase on the Tc of the phospholipids, resulted in a reduction on the I.E. from 38 to 13% and to a final TPD to lipid ratio from 10 to 3 nmol per µmol of lipid, respectively.

The TPD was also incorporated in a lipid composition containing PEG that is widely recognized, from *in vivo* studies, to reduce plasma proteins adsorption at liposome surface [35,36]. Incorporation parameters were lower than the ones observed for formulation 1: 33% and 12 nmol per µmol of lipid, for the I.E: and final TPD to lipid ratio, respectively.

### Stability of TPD formulations in human plasma

To better understand the *in vivo* performance of this prodrug, the hydrolysis rate was assessed after incubation in human plasma at 37°C. Blood serum and plasma are known to contain a wide range of enzymes that are able to catalyze the hydrolysis of amides and particularly this prodrug [37]. A molecule that it is designed to act on cancer cells must present a good ability to spread among cell membranes, and to present a half-life, in plasma medium, long enough until reaching the target site.

The chemical stability of TPD in free and liposomal forms was determined by evaluating the hydrolysis rate of the prodrug as well as the appearance of MMT and of the amine. As an example it is shown in Figure 3 a graphical representation of the time course for the disappearance of TPD and formation of MMT and amine.

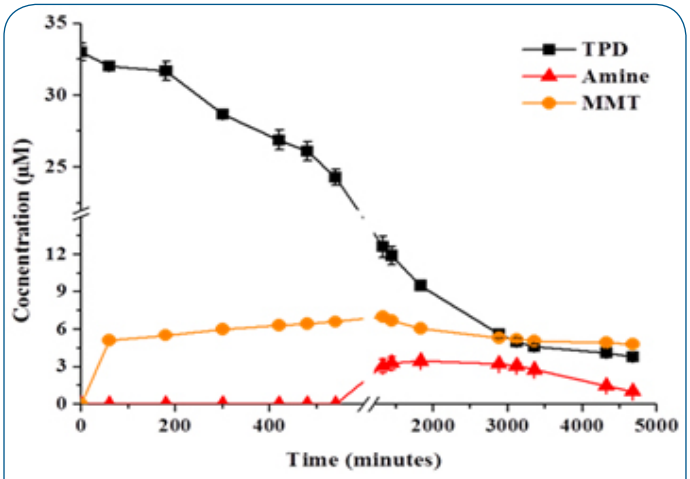



**Figure 3:** Stability of TPD incorporated in liposomes in human plasma. Time course for the disappearance of TPD and formation of MMT and amine. Incubation at 37°C of TPD liposomes in human plasma 80% (v/v) with an initial theoretical concentration of 35 µM. The results express the mean ± S.D. of at least three independent experiments

The apparent first-order hydrolysis rate constants of TPD at 37°C were determined by plotting the logarithm of prodrug concentration as a function of time and the relation between rate constant and $k_{obs}$ led to determination of half-life. In Table 4 are shown the respective half-lives and the correlation coefficient ($R^2$) obtained for each formulation under study.

The hydrolysis rate, of TPD incorporated in liposomes presented half-lives of around 1000 min particularly for formulation 3, which contains PEG in the lipid composition. The presence of PEG is known to decrease the adsorption of plasma proteins at liposome surface [28,35]. Nevertheless, besides the hydrolysis rate other important properties namely cyotoxic effect in melanoma

**Table 4:** Hydrolysis rate of TPD in free and liposomal forms in the presence of 80% human plasma, at 37°C

| Formulation | half-life (min) | Correlation coefficient ($R^2$) |
|---|---|---|
| **1** | 834 ± 45 | 0.9624 |
| **2** | 719 ± 36 | 0.9896 |
| **3** | 940 ± 55 | 0.9551 |
| **4** | 677 ± 66 | 0.9422 |
| **5** | 770 ± 50 | 0.9328 |
| **Free TPD** | 1155 ± 19 | 0.9700 |

TPD initial theoretical concentration of 35 μM

The results express the mean ± S.D. of at least three independent experiments

cells, should be taken into account for the selection of prodrugs as therapeutic candidates [8,22]. We must also emphasize that the incorporation of prodrugs in liposomes may solve solubility problems associated to these molecules eliminating the use of toxic solvents [15,19,36]. In previous work, the half-life for this prodrug in the free form was much lower: 319 min [13,20] but the experimental conditions were not the same as the ones used in the present work. Nevertheless, the present works enabled the comparison of TPD in free and liposomal forms using the same procedures.

### *In vitro* tests in a malignant human melanoma cell line

To evaluate the anti-tumor activity of TPD in free and liposomal forms, *in vitro* studies were performed using a human melanoma cell line. In previous work, the cytotoxic effect of this TPD using different human melanoma cell lines was investigated [13,20]. In particular, the melanoma cell line, MNT-1, showed a high tyrosinase specific activity and so it was selected in the present work.

In the present *in vitro* tests, formulations 1 and 3 were selected. The reason for choosing these TPD formulations was based on high incorporation parameters and stability in human plasma observed. Moreover, the formulation 3, characterized by the presence of PEG at liposome surface, will present good perspectives when administered *in vivo* due to the long circulation properties in blood stream and consequently enabling higher accumulation in tumor sites [15,28,35].

The cytotoxic effect of TPD in free and liposomal forms was performed using increased concentrations ranging from 7.5 to 125 μM. The negative control consisting only on MNT-1 cells, corresponded to 100% of cell viability. The cytotoxic effect was evaluated after an incubation period of 24, 48 and 72 h. A dose dependent decrease in the MNT-1 cell viability was observed, particularly for TPD in liposomal form. As an example in Figure 4 is shown, comparatively, the cellular viability of MNT-1 cells 24 h after incubation with TPD in free form (Free TPD), incorporated in liposomes (Lip TPD). Unloaded liposomes were also included in this study using the same lipid concentrations as those tested for TPD liposomes. They did not exert any reduction on cellular viability of MNT-1 cells, ensuring that the cytotoxic effect observed in TPD liposomes was not attributed to the phospholipid itself. Regarding Free TPD the cellular viability

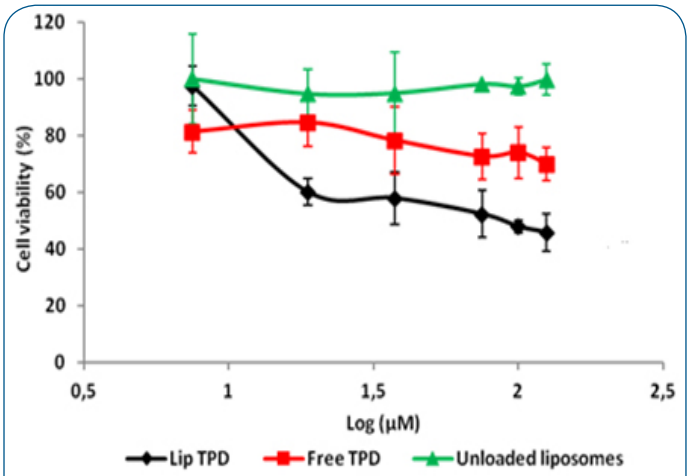



**Figure 4:** Graphical representation of MNT-1 cells viability 24 h post-incubation with TPD in free and liposomal forms as well as unloaded liposomes. Tested concentrations ranged from 7.5 to 125 μM. Lipid composition PC

was always higher than 75% for all the tested concentrations. However for the TPD incorporated in liposomes a high cytotoxic effect was achieved with an $IC_{50}$ of 87 μM.

As it was not possible to determine the $IC_{50}$ for the free TPD using these experimental conditions, longer incubation periods were tested, 48 and 72 h. The cellular viability of MNT-1 cells was evaluated after incubation of TPD in free form or incorporated in PC or PC:PEG (formulations 1 and 3). In Table 5 are shown the respective $IC_{50}$ values obtained.

According to the obtained results, TPD in the free form was not cytotoxic enough to reduce more than 50% of cells and thus it was not possible the determination of the $IC_{50}$ using TPD concentrations up to 125 μM. The incorporation of TPD in liposomes was able to potentiate the cytotoxicity of this prodrug as $IC_{50}$ values ranging from 13 to 22 μM were achieved for TPD formulations 1 and 3, respectively, 72 h after incubation with MNT-1 cells. The cytotoxic effect of TPD incorporated in liposomes was higher in comparison with TMZ, a triazene prodrug in clinical use. The superior anti-tumor effect of TPD following its association to liposomes is in accordance with literature [38,39]. This is due to an increased bioavailability after transport of liposomes to cytoplasm, where degradative enzymes breakdown the liposomal membrane and release the prodrug [40].

**Table 5:** Influence of TPD formulations on MNT-1 cell viability

| Formulation | $IC_{50}$ (μM) Incubation time (h) | |
|---|---|---|
| | **48** | **72** |
| **1** | 16 ± 1 | 13 ± 1 |
| **3** | 68 ± 3 | 22 ± 2 |
| **Free TPD** | > 125 | > 125 |
| **Temozolomide** | > 75 | > 75 |

Tested concentrations ranged from 7.5 to 125 μM. Formulations 1 and 3 correspond to TPD incorporated in PC and in PC:PEG liposomes, respectively

## Conclusions

Several alternatives to solve problems associated to melanoma therapies, such as low concentration in tumors sites, lack of selectivity between tumor and normal cells led us to consider the urgent need of novel molecules and/or novel drug delivery systems.

In the present work the incorporation of a newly synthesized triazene prodrug in liposomes was able to potentiate its cytotoxic effect against a human malignant melanoma cell line. Moreover, this antitumor activity was superior when compared to TMZ a triazene prodrug in clinical use. These preliminary results constitute a promising approach for melanoma therapy as liposomes were able to provide an effective treatment for this aggressive pathology by improving solubility of hydrophobic molecules and concomitant increased concentration in tumor cells.

The establishment of a murine melanoma model to confirm the *in vitro* studies constitutes the following step.

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